

This Page Is Inserted by IFW Operations  
and is not a part of the Official Record

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning documents *will not* correct images,  
please do not report the images to the  
Image Problem Mailbox.**

**REMARKS**

Reconsideration of this application is respectfully requested.

Claims 29-31 were rejected under 35 U.S.C. § 101 and under 35 U.S.C. § 112, first paragraph, for allegedly not being supported by a well-established utility. Although the Examiner concedes that the prior art describes using immune complexes to induce immune responses, the Examiner contends that this utility is not disclosed in the instant application. (Office Action at 1-2.) Applicants traverse the rejection.

The lack of a specific statement of utility in the specification does not negate utility *per se*. M.P.E.P. 2107.02(II)(B). Rather, if an invention has a well-established utility, rejections under 35 U.S.C. § 101 and 35 U.S.C. § 112, first paragraph, based on lack of utility should not be imposed. *Id.* (citing *In re Folkers*, 344 F.2d 970, 145 U.S.P.Q. 390 (C.C.P.A. 1965)).

Applicants' claimed immune complexes have a well-established utility, namely, they can be used to generate immune responses. The Examiner has not asserted that applicants' immune complexes could not be used in this well-established utility. In fact, the Examiner has recognized that this utility for immune complexes can be found in the prior art. (Office Action at 1-2.)

There is no reason to believe that applicants' immune complexes could not be used to induce immune responses. Accordingly, the rejection of applicants' claimed immune complexes for lacking utility is improper. *See id.*

Furthermore, applicants provide herewith objective evidence (Exhibits 1-3) that immune complexes were well-known to be useful for the generation of immune responses prior to the

filing date of this application. In Nishi, 1970 (Exhibit 1), immune complexes (antibody- $\alpha$ -globulin) were used to immunize a horse. Nishi at 2507, col. 2. The serum from the horse showed a highly-specific reaction for  $\alpha$ -globulin. *Id.* at 2509, col. 1. In addition, Higgins, 1980, (Exhibit 2), indicates that immune complexes can be used “as immunogens to elicit the formation of a more highly specific antisera.” Higgins at 889, col. 1. In this paper, agarose-immobilized immune complexes were used to immunize a rabbit. Higgins at 889, col. 2. The antiserum generated against the immune complexes was higher titer than the original antiserum. *Id.* Moreover, Eager et al., 1983 (Exhibit 3), indicate that immunization with immune complexes can be an effective way to produce monoclonal antibodies. Eager et al. at 157. This paper indicates that immune complexes acted as effective immunogens in both mice and rats, and the advantages of using this method for immunization are discussed. *Id.* at 163. The skilled artisan would immediately appreciate that applicants’ immune complexes were likewise useful. Thus, applicants’ claimed invention has a well-established utility. Accordingly, applicants respectfully request withdrawal of the rejections.

Claims 29-31, 39, 40, and 45-49 were rejected under 35 U.S.C. § 112, first paragraph, for allegedly containing subject matter that was not described in the specification in such a way as to reasonably convey to the skilled artisan that the inventors had possession of the claimed invention at the time the application was filed. The Examiner does not dispute that the Declaration of Dr. Cohen under 37 C.F.R. § 1.132 demonstrates that the skilled artisan would be capable of following the teachings of the disclosure to prepare the claimed invention. However,

the Declaration is deemed insufficient by the Examiner because “[t]here is nothing in the disclosure to suggest to the skilled artisan that applicants actually prepared the claimed immune complexes or antibodies.” (Office Action at 4-5.) The Examiner indicates that applicants may obviate the rejection by providing scientific evidence that the claimed antibodies and immune complexes were actually generated. (Office Action at 4.) Applicants traverse the rejection.

The Examiner’s insistence on the actual preparation of the claimed immune complexes or antibodies (i.e., a working example) to fulfill the written description requirement of 35 U.S.C. § 112, first paragraph, is contrary to current legal precedent. As explained in applicants’ January 21, 2003, Amendment and Response to Paper No. 51, decisions by both the Court of Appeals for the Federal Circuit (*Gould v. Quigg*) and the Supreme Court (*Pfaff v. Wells Electronics, Inc.*) dictate that a working example is ***not required*** for applicants to fulfill the written description requirement of 35 U.S.C. § 112, first paragraph. Applicants respectfully request that the Examiner explain how his requirement for a working example can be maintained in view of these two cases.

Moreover, the Examiner’s position is untenable in view of the recent decision in *Noelle v. Lederman*, 355 F.3d 1343, 1349, 69 U.S.P.Q.2d 1508, 1514 (Fed. Cir. 2004). In *Noelle*, the Court of Appeals for the Federal Circuit found that an applicant can claim an antibody by its binding affinity to an antigen if the applicant has disclosed a fully characterized antigen either by its structure, formula, chemical name, or physical properties, or by depositing the protein in a public depository. *Id.* Thus, in *Noelle*, it was not necessary for the applicant to have “actually

prepared” the antibody to claim it. It was sufficient to have fully described the antigen, and such a description could be accomplished through a deposit of the antigen. The same is true of applicants’ claimed antibodies and immune complexes. The Examiner’s requirement for applicants to have “actually prepared” the antibodies and immune complexes to claim them (i.e., a working example) runs counter to the decision in *Noelle*, and is in error.

According to *Noelle*, applicants do not need to have a working example of making antibodies against the claimed antigens to meet the written description requirement. Rather, they can meet the requirements of 35 U.S.C. § 112, first paragraph, by disclosing a “fully characterized antigen” either by its structure, formula, chemical name, or physical properties, or by depositing the protein in a public depository. *See id.* Applicants’ specification fulfills this requirement. Applicants deposited isolates of HIV-1 at the Collection Nationale des Cultures de Micro-organismes (specification at 27), and described how to propagate HIV-1 and isolate the antigens recited in claims 29-31, 39, 40, and 45-49 (specification at 10-13). Since the deposited virus contains the recited antigens, applicants’ deposit should be viewed as fulfilling the written description requirement of 35 U.S.C. § 112, first paragraph, for antibodies against these “fully characterized antigens.” *See Noelle*, 355 F.3d at 1349, 69 U.S.P.Q.2d at 1514. Accordingly, applicants respectfully request withdrawal of the rejection.

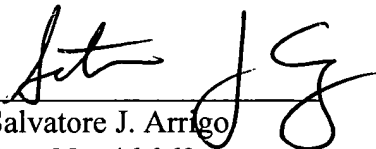
Applicants respectfully submit that this application is in condition for allowance. In the event that the Examiner disagrees, he is invited to call the undersigned to discuss any outstanding issues remaining in this application in order to expedite prosecution.

Please grant any extensions of time required to enter this response and charge any additional required fees to our deposit account 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,  
GARRETT & DUNNER, L.L.P.

By: \_\_\_\_\_

  
Salvatore J. Arrigo

Reg. No. 46,063

Tel: 202-408-4000

Fax: 202-408-4400

E-mail: [arrigos@finnegan.com](mailto:arrigos@finnegan.com)

Dated: May 18, 2004

SIDNEY WEINHOUSE, *Editor*

MARGARET FOTI, *Managing Editor*

*Editorial Assistants*

TINA ANTONINI

BARBARA CANTARELLA

JILL FILLER

*Associate Editors*

RENATO BASERGA  
NATHANIEL I. BERLIN  
JOHN J. BIESELE  
JOSEPH H. BURCHENAL  
HARRIS BUSCH  
KELLY H. CLIFTON  
ALBERT J. DALTON  
EUGENE D. DAY  
KENNETH B. DEOME  
EMMANUEL FARBER  
GILBERT H. FRIEDEL  
HARRY V. GELBOIN

ABRAHAM GOLDIN  
KARL E. HELLSTRÖM  
RUSSELL HILF  
ERICH HIRSCHBERG  
ROBERT B. HURLBERT  
MARVIN KUSCHNER  
GERALD A. LEPAGE  
HERMANN LISCO  
ROBERT LOVE  
NATHAN MANTEL  
JOSEPH L. MELNICK  
DAN H. MOORE  
CHARLES A. NICHOL

PETER C. NOWELL  
FRED S. PHILIPS  
HENRY C. PITOT  
DAVID P. RALL  
R. WAYNE RUNDLES  
ALBERT SEGALOFF  
MICHAEL B. SHIMKIN  
JESSE L. STEINFELD  
ARTHUR C. UPTON  
GEORGE WEBER  
JOHN H. WEISBURGER  
CHARLES E. WENNER  
SUMNER WOOD, JR.

*Editorial Advisory Board*

CREED W. ABELL  
SAMUEL ABRAHAM  
JOSEPH C. ARCOS  
JOHN C. BAILAR, III  
M. EARL BALIS  
W. RAY BRYAN  
WALLACE H. CLARK  
BAYARD D. CLARKSON  
ROGER DAOUST  
CLYDE J. DAWE  
VITTORIO DEFENDI  
SHELDON M. EPSTEIN  
EMIL FREI, III  
ROBERT A. GOOD

THEODORE S. HAUSCHKA  
CHARLES C. IRVING  
NATHAN KALISS  
WERNER H. KIRSTEN  
MONTAGUE LANE  
ELIZABETH H. LEDUC  
MORTIMER L. MENDELSON  
ENRICO MIHICH  
JOHN B. MOLONEY  
PHILIP O'B. MONTGOMERY, JR.  
HARVEY M. PATT  
G. BARRY PIERCE  
ALAN S. RABSON

HERBERT J. RAPP  
JAY S. ROTH  
KATHERINE K. SANFORD  
ALAN C. SARTORELLI  
EMMA SHELTON  
M. MICHAEL SIGEL  
EDWARD A. SMUCKLER  
SAM SOROF  
HERMAN D. SUIT  
VICTOR A. TRIOLO  
BENJAMIN L. VAN DUUREN  
EUGENE J. VAN SCOTT  
ERNEST WYNDER  
WASLEY D. YUSHOK

CANCER RESEARCH is sponsored by the American Association for Cancer Research, Inc.; The Jane Coffin Childs Memorial Fund for Medical Research; The Elsa U. Pardee Foundation; The American Cancer Society; the National Cancer Institute, Public Health Service; and the Damon Runyon Memorial Fund for Cancer Research, Inc.

**NOTICE TO MEMBERS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH**

*Officers for 1970-1971*

*President:* JAMES F. HOLLAND, Roswell Park Memorial Institute, Buffalo, New York 14203  
*Vice President:* EMIL FREI, III, The University of Texas M. D. Anderson Hospital and Tumor Institute, Houston, Texas 77025

*Secretary-Treasurer:* HUGH J. CREECH, Institute for Cancer Research, Fox Chase, Philadelphia, Pennsylvania 19111

The annual dues of active members of the American Association for Cancer Research include a subscription to the Journal CANCER RESEARCH. The regular annual subscription price to CANCER RESEARCH for members of the Association is \$12.50 per annual volume. Payment of dues and changes of address of members of the Association should be sent promptly to Dr. Creech.

Papers submitted for publication and all other communications for the attention of the editor should be sent to Dr. Sidney Weinhouse, *Editor*, CANCER RESEARCH, Fels Research Institute, Temple University School of Medicine, Beury Bldg., 3701 N. Broad St., Philadelphia, Pennsylvania 19140. Telephone: Area Code 215-BA 3-2150.

**NOTICE TO GENERAL SUBSCRIBERS**

CANCER RESEARCH is published monthly, one volume per year, for Cancer Research, Inc., by The Williams & Wilkins Company, 428 E. Preston St., Baltimore, Maryland 21202. Subscriptions include the Proceedings of the American Association for Cancer Research issued in March of each year. Except for members of the American Association for Cancer Research, all subscriptions are payable in advance to the publisher of the Journal, The Williams & Wilkins Co., to which all business communications, remittances (in United States currency or its equivalent), and subscription orders should be sent. Non-members of the American Association for Cancer Research, Inc., may subscribe to the Journal CANCER RESEARCH at the rate of \$50.00 per annual volume, including postage, beginning with the 1971 issues. When available, single copies will be sold for \$7.00.

Notify the publisher of changes of address 60 days in advance, giving both the old and new addresses. Copies undeliverable because of address changes will be destroyed.

**BACK ISSUES OF THE JOURNAL**

Copies of back stock of the Journal CANCER RESEARCH for the two years preceding the current year may be ordered from The Williams & Wilkins Co., address above. All earlier issues may be obtained from Walter J. Johnson, Inc., 111 Fifth Ave., New York, New York 10003. Copies of Part 2 (The Cancer Chemotherapy Screening Data Supplements) may be obtained from the Cancer Chemotherapy National Service Center, Bethesda, Maryland 20014.

Second-class postage paid at Baltimore, Maryland.

Copyright 1970 by Cancer Research, Inc.

# Isolation and Characterization of a Human Fetal $\alpha$ -Globulin from the Sera of Fetuses and a Hepatoma Patient

Shinzo Nishi

Department of Biochemistry, School of Medicine, Hokkaido University, Sapporo, Japan

## SUMMARY

It has been demonstrated that fetal serum contains an  $\alpha$ -globulin which is not present in normal adults. This fetal  $\alpha$ -globulin has become the subject of great interest because it appears again specifically in the sera of hepatoma patients. It was isolated from the serum of a patient with hepatoma as well as from fetuses by an immunochemical method. Physicochemical and chemical properties of the two preparations were studied, and essentially identical results were obtained.

## INTRODUCTION

Fetal serum of many mammalian species, including humans, contains an  $\alpha$ -globulin which is not present in the serum of the normal adult (4, 7, 12, 22, 32). In humans, this fetal-specific protein is found in the serum during the gestational period and is variously called  $\alpha_f$ -globulin, postalbumin,  $\alpha$ -fetoprotein,  $\alpha$ -fetoglobulin, Substance X, etc. (1, 3, 4, 7, 11).

In 1963, Abelev *et al.* (2) first reported that transplantable mouse hepatomas synthesized and secreted into the blood an  $\alpha$ -globulin which was immunologically identical to  $\alpha_f$ -globulin. Similar observations were made on rats (13) and monkeys (15) with chemically induced primary hepatomas.

Tatarinov and several other investigators (1, 3, 18, 23, 29–31) have shown that  $\alpha_f$ -globulin is present in 50 to 80% of patients with hepatomas.

Previous investigations of the physicochemical and chemical natures of these fetal  $\alpha$ -globulins have dealt almost exclusively with the calf (22, 25, 26) and rat (17, 27), but to the present human  $\alpha_f$ -globulin has not been extensively studied.

The present paper deals with the isolation and characterization of human  $\alpha_f$ -globulin from human fetuses and a hepatoma patient.

## MATERIALS AND METHODS

### Fetal Serum

Human fetuses, ranging from 5 to 7 months gestational period, were obtained by abortion, and blood was taken by heart puncture. Sera of 10 fetuses were pooled.

### Hepatoma Patient Serum

Serum was obtained from a 15-year-old male patient with histologically confirmed hepatocellular carcinoma.

### Production of Antisera

**Absorption.** Rabbits were given s.c. injections of 0.1 ml of the pooled fetal serum emulsified in an equal volume of Freund's complete adjuvant at weekly intervals for 3 injections. The rabbits were bled 2 weeks after the last injection, and the antisera against fetal serum were collected and pooled. The antiserum was absorbed with pooled normal adult serum from 10 persons adding 3 ml of the pooled serum to 1 ml of the antiserum. The absorbed antiserum thus obtained was demonstrated to be specific for  $\alpha_f$ -globulin.

**Immunization with Antigen-Antibody Precipitate.** The absorbed rabbit antiserum specific for  $\alpha_f$ -globulin was mixed with the hepatoma patient serum at optimal precipitation proportion. The solution was allowed to react at 37° for 60 min, and formed antigen-antibody precipitate was collected by centrifuging at 3,000 rpm for 15 min and washed with 0.15 M NaCl solution 3 times in the cold. The precipitate was then dissolved in 5 volumes of 0.10 M glycine-HCl buffer, pH 1.8, and the solution was centrifuged at 10,000 rpm for 15 min to remove insoluble material. The pH of the solution was then neutralized to 7.0 with 0.4 M Na<sub>2</sub>HPO<sub>4</sub>, whereupon the antigen-antibody precipitate reformed. This procedure, i.e., dissolving the antigen-antibody precipitate in acid and precipitating the antigen and the antibody by neutralization, was repeated twice as shown in Chart 1. By this treatment, impurities other than antigen and antibody appeared to be completely removed.

A horse was given an injection s.c. in the side of the neck with the washed precipitate emulsified in Freund's complete adjuvant at intervals of 10 days for 3 injections. For each injection, the precipitate prepared from 1 ml of the hepatoma patient serum (about 0.5 mg of the antigen) was used. Two weeks after the last injection, the horse was bled from the carotid and the antiserum was obtained.

### Purification of $\alpha_f$ -Globulin from Antigen-Antibody Precipitate

$\alpha_f$ -Globulin was purified both from the hepatoma patient serum and the pooled fetal serum. Antigen-antibody precipi-

Received April 20, 1970; accepted June 16, 1970.



Chart 1

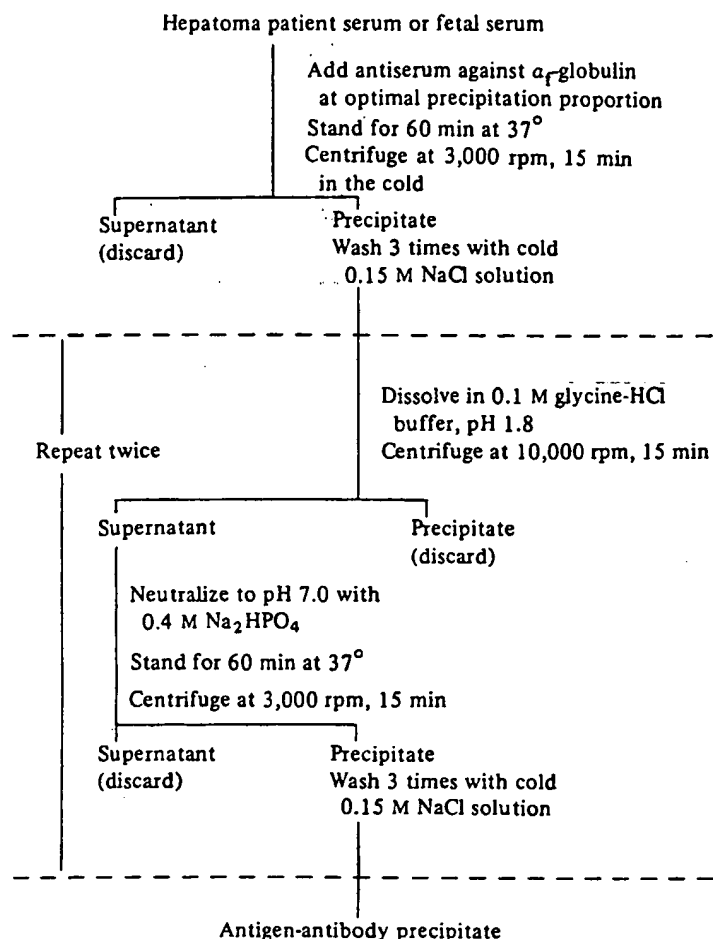


Chart 1. Preparation of antigen-antibody precipitate.

tate was prepared by mixing the horse antiserum and either the fetal serum or the hepatoma patient serum according to the procedure shown in Chart 1.

The precipitate was dissolved in an equal volume of 0.10 M glycine-HCl buffer, pH 1.8, and subjected to gel filtration on Sephadex G-150 column (2.6 x 95 cm) equilibrated with the same buffer. A 5-ml volume of the solution, about 150 mg of antigen-antibody precipitate, was applied to one column. The dissociated antigen and antibody were eluted at a flow rate of 20 ml/hr. The spectrophotometric absorption of the eluate at 280 mμ was constantly monitored, and the eluate was collected in 5-ml fractions. Each fraction was neutralized with 0.4 M Na<sub>2</sub>HPO<sub>4</sub> and the presence of α<sub>f</sub>-globulin was determined by radial immunodiffusion test as described below. α<sub>f</sub>-Globulin containing fractions were pooled and concentrated to 3 ml by ultrafiltration and subjected again to gel filtration to remove a small amount of contaminating antibody. In the 2nd gel filtration, the acidic buffer used in the 1st gel filtration was replaced with phosphate-buffered 0.15 M NaCl solution, pH 7.0.

The purified α<sub>f</sub>-globulin thus obtained was dialyzed exhaustively against redistilled water and lyophilized.

## Double Immunodiffusion, Immunoelectrophoresis, and Radial Immunodiffusion

The double diffusion method was that described by Ouchterlony (21). The immunoelectrophoretic method was that described by Grabar and Williams (14). The radial immunodiffusion method was that described by Mancini *et al.* (19). A 1.0% agarose gel in 0.05 M barbital buffer, pH 8.6, was used in the above methods.

## Disc Electrophoresis

The disc electrophoretic method was that described by Davis (9).

## Physicochemical Analyses

**Sedimentation Coefficient.** α<sub>f</sub>-Globulin dissolved in 0.15 M NaCl solution was examined with a Spinco Model E ultracentrifuge at 56,100 rpm. The sedimentation coefficient was measured at various concentrations (1.4 to 0.4%) and extrapolated to zero concentration.

**Diffusion Coefficient.** The diffusion coefficient of a 0.6% α<sub>f</sub>-globulin solution was measured in a synthetic boundary cell at 15,220 rpm for 55 min by the method of Schachman as modified by Kawahara (16). Molecular weight was calculated from the equation of Svedberg (28). A partial specific volume calculated from amino acid composition (20) was used in the above calculations.

**Extinction Coefficient.** The ultraviolet absorption of α<sub>f</sub>-globulin was measured in redistilled water with a Hitachi Perkin-Elmer 139 UV-VIS spectrophotometer. The concentration was determined by drying aliquots to constant weight at 100° over P<sub>2</sub>O<sub>5</sub> in a vacuum.

## Chemical Analyses

**Nitrogen and Sulfur.** Nitrogen was determined by a micro-Dumas method. Sulfur was determined by the method of Schöniger (24).

**Nonnitrogenous Sugars.** Nonnitrogenous sugars were determined by the Winzler orcinol-sulfuric acid reaction (10). Glucose was used as standard sugar.

**Amino Acid Composition.** Amino acid analyses were performed with a Hitachi model KLA-3 automatic amino acid analyzer (6). Hydrolysis was performed in 6 N HCl in an evacuated, sealed tube at 110° for 24 hr. After hydrolysis HCl was removed in a vacuum rotor at 35–40°. Tryptophan and tyrosine were determined from the ultraviolet absorption spectrum of α<sub>f</sub>-globulin in 0.1 N NaOH by the method of Bencze and Schmid (5).

## RESULTS

**Antisera.** Rabbit antiserum against fetal serum developed about 10 precipitation lines against either fetal or normal adult sera by immunoelectrophoresis. When absorbed, the antiserum gave only a single precipitation line against the fetal sera in the region between albumin and α<sub>1</sub>-globulin bu

did not react with the normal adult sera. An example is shown in Fig. 1. Against hepatoma patient sera, the antiserum gave also a single precipitation line fusing completely with the fetal serum precipitation line. An example is shown in Fig. 2.

The horse antiserum prepared by immunization with the antigen-antibody precipitate also showed the highly specific reaction for  $\alpha_f$ -globulin against sera from patients with hepatoma and fetuses. This antiserum also contained antibody against rabbit immunoglobulin, but this did not interfere with the above reaction.

**Clinical Data.** A few clinical investigations were carried out with the horse antiserum. The prevalence of  $\alpha_f$ -globulin was as follows.  $\alpha_f$ -Globulin was detected by radial immunodiffusion test in sera of 23 out of 31 patients with primary liver cancer. In 20 histologically confirmed cases, 16 showed positive reactions. Sera of 500 patients with noncancerous liver diseases, hepatitis, cirrhosis, abscess, etc., and sera of 100 patients with malignant tumors of nonliver origin, including liver metastases, gave negative reactions with one exception. This positive serum was from a patient with gastric cancer with massive liver metastasis. Details of the clinical study will be presented elsewhere.

**Purification.** Antigen-antibody precipitate prepared from either the pooled fetal serum or the hepatoma patient serum was dissolved and subjected to gel filtration. From the 1st gel filtration, 3 peaks were obtained (Chart 2a). Peak 1 gave a negative test for antibody and antigen by immunodiffusion test. In addition, this fraction precipitated almost quantitatively when neutralized and appeared to consist of antigen-antibody complex. Peak 2 gave a positive test for antibody and a negative test for antigen and appeared to consist of antibody. Peak 3 gave a negative test for antibody and a positive test for antigen. Radial immunodiffusion test showed that this peak consisted of  $\alpha_f$ -globulin.

Peak 3 was again subjected to gel filtration at neutral pH. On the 2nd gel filtration, contaminating antibody formed a soluble antigen-antibody complex because of antigen excess, and 2 peaks were obtained (Chart 2b). Both peaks gave a positive test for antigen and a negative test for antibody. The position of Peak 5 in the elution pattern was identical to that of  $\alpha_f$ -globulin in the elution pattern of the fetal serum or the hepatoma patient serum. Peak 5 was dialyzed and lyophilized.

From 30 ml of the pooled fetal serum, 30 mg of  $\alpha_f$ -globulin were prepared; from 50 ml of the hepatoma patient serum, 20 mg of  $\alpha_f$ -globulin were prepared. The yield of this method determined by radial immunodiffusion test was approximately 70%. The 2  $\alpha_f$ -globulin preparations were homogeneous both by electrophoresis and ultracentrifugation. Fig. 3 shows the pattern of cellulose acetate membrane electrophoresis, and Fig. 4 shows the pattern of disc electrophoresis. By immunoelectrophoresis, both preparations gave a single precipitation line against antiserum to  $\alpha_f$ -globulin but did not react with hyperimmune serum to adult human serum (Hyland Laboratories, Los Angeles, Calif.) (Fig. 5). An ultracentrifugal pattern shown in Fig. 6 indicates a high degree of homogeneity.

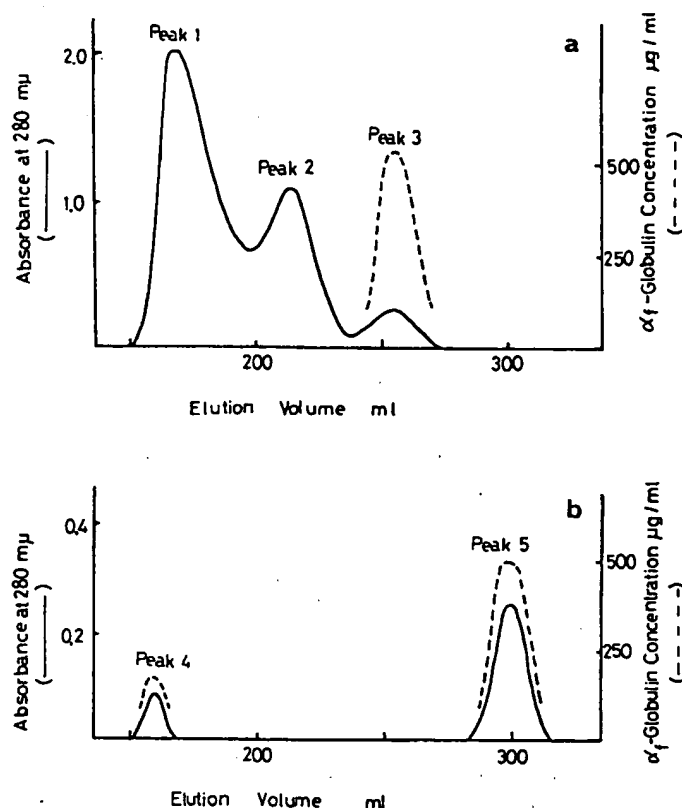


Chart 2. Purification of  $\alpha_f$ -globulin from antigen-antibody precipitate prepared from a hepatoma patient serum by gel filtration on Sephadex G-150 column. The 1st gel filtration (a) was performed as follows. Eluate, 0.1 M glycine-HCl buffer, pH 1.8; sample, 5 ml of antigen-antibody precipitate (about 150 mg) dissolved in the same buffer; flow rate, 20 ml/hr; column dimensions, 2.6 x 95 cm. The protein concentration was determined by absorbance at 280 m $\mu$ . The  $\alpha_f$ -globulin concentration was determined by radial immunodiffusion test with the use of antiserum against  $\alpha_f$ -globulin. The 2nd gel filtration (b) was performed as follows. Eluate, phosphate-buffered 0.15 M NaCl solution, pH 7.0; sample, Peak 3 in the first experiment neutralized and concentrated to 3 ml. Other conditions were similar to the first experiment. Peak 4 consisted of soluble antigen-antibody complex and Peak 5 consisted of antigen. Peak 5 was homogeneous both by electrophoresis and by ultracentrifugation.

Virtually the same result was obtained with the antigen-antibody precipitate from fetuses.

**Physicochemical and Chemical Analyses.** Analytical data of  $\alpha_f$ -globulin preparations both from fetal serum and the hepatoma patient serum were the same, within the errors of the experimental methods.

Some physicochemical data are shown in Table 1. The sedimentation coefficients showed a slight concentration dependency as shown in Chart 3. The partial specific volume which was calculated from amino acid composition and did not include that of carbohydrate portion was 0.726 ml/g (fetus).

$\alpha_f$ -Globulin dissolved in redistilled water gave an ultraviolet absorption spectrum characteristic for a protein with a maximum at 278 m $\mu$ . The extinction coefficient  $E_{1\%}^{1\text{cm}}$  (278 m $\mu$ ) was 5.30 (fetus).

Table 1

Physicochemical properties of  $\alpha_f$ -globulin

Source of $\alpha_f$ -globulin	$s_{20,w}^0$ [ $10^{-13}$ sec (S)]	$D_{20,w}^0$ ( $10^{-7}$ sq cm/sec)	Molecular weight	$E_{1\%}^{1\text{cm}}$ (278 $m\mu$ )	$V$ (ml/g)
Hepatoma patient	4.50	ND <sup>a</sup>	ND	5.26	0.727
Fetus	4.50	6.18	64,600	5.30	0.726

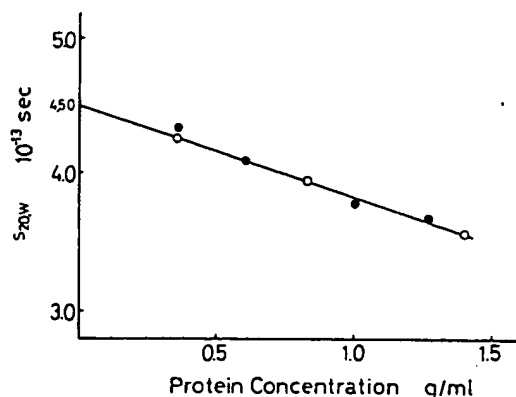
<sup>a</sup>Not done.

Chart 3. Effect of protein concentration on the sedimentation coefficient of  $\alpha_f$ -globulin. The determination was carried out at various concentrations in 0.15 M NaCl solution. The value extrapolated to zero concentrations was 4.50 S ( $4.50 \times 10^{-13}$  sec).  $\circ$ ,  $\alpha_f$ -globulin prepared from a hepatoma patient;  $\bullet$ ,  $\alpha_f$ -globulin prepared from fetuses.

The contents of nitrogen, sulfur, and nonnitrogenous sugars are shown in Table 2.

The amino acid composition is shown in Table 3. Tryptophan and tyrosine were determined spectrophotometrically as described above. Good agreement was found between the values of tyrosine determined by the automatic amino acid analyzer method and by the spectrophotometric method.

The content of the peptide portion of this protein calculated from the sum of all the amino acid residues was approximately 96%.

## DISCUSSION

A marked interest has been taken in this fetal-specific  $\alpha$ -globulin in recent years because its reappearance with the

Table 2

Chemical composition of  $\alpha_f$ -globulin

Component	Content (1%)	
	Hepatoma <sup>a</sup>	Fetus <sup>b</sup>
Nitrogen	14.9	14.7
Sulfur	1.8	1.7
Nonnitrogenous sugars	3	3

<sup>a</sup>Values determined for  $\alpha_f$ -globulin from a hepatoma patient.<sup>b</sup>Values determined for  $\alpha_f$ -globulin from fetuses.

Table 3

Amino acid composition of  $\alpha_f$ -globulin

Amino acids were determined on a Hitachi model KLA-3 automatic amino acid analyzer except tryptophan, which was determined spectrophotometrically.

Amino acid	Hepatoma <sup>a</sup>	Fetus <sup>b</sup>	Amino acid	Hepatoma	Fetus
Asparagine	88 <sup>c</sup>	87	Methionine	10	8
Threonine	63	65	Isoleucine	46	44
Serine	64	66	Leucine	96	94
Glutamine	186	197	Tyrosine	29	28
Proline	39	38	Phenylalanine	52	48
Glycine	48	47	Lysine	63	64
Alanine	87	90	Histidine	21	22
Half-cystine	24	20	Arginine	30	31
Valine	51	48	Tryptophan	3	3

<sup>a</sup>Values determined for  $\alpha_f$ -globulin from a hepatoma patient.<sup>b</sup>Values determined for  $\alpha_f$ -globulin from fetuses.<sup>c</sup>Values are expressed as moles/1000 moles of amino acids. Good agreement was found between the two values.

primary cancer of the liver not only is of great diagnostic and prognostic value but may offer a new approach to the interpretation of the process of carcinogenesis. However, to date there have been relatively few studies on the physicochemical and chemical structure of this protein, particularly of human  $\alpha_f$ -globulin.

Bergstrand and Czar (8) obtained a fraction containing  $\alpha_f$ -globulin and albumin by electrophoretic separation of fetal serum. Ultracentrifugal analysis of this fraction showed a single but slightly asymmetric peak. He estimated that this protein has a slightly smaller sedimentation coefficient than that of albumin, which value is 4.6 S.

Gitlin and Boesman (11) estimated that the sedimentation coefficient of this protein is approximately 5.0 S by ultracentrifugation of fetal serum in a sucrose density gradient. He also observed that by gel filtration of fetal serum on Sephadex G-200 this protein was eluted between transferrin and albumin, the molecular weights of which are 69,000 and 90,000, respectively. These observations are in good agreement with the present data.

The presence of fetal-specific  $\alpha$ -globulin has been reported in many mammalian species. Among these, only calf fetuin, rat postalbumin, and rat antigen LA were isolated in a high degree of purity and characterized.

Compared to human  $\alpha_f$ -globulin, calf fetuin has a smaller sedimentation coefficient (3.47 S), smaller molecular weight (48,400), higher hexose content (8.3%), and quite different amino acid composition (25, 26), and seems to be distinct from human  $\alpha_f$ -globulin.

On the other hand, rat postalbumin purified from fetal plasma by Kirsch *et al.* (17) has a sedimentation coefficient of 4.43 S, a diffusion coefficient of  $6.25 \times 10^{-7}$  sq cm/sec, and a molecular weight of 64,800. These values are in close agreement to the present data, and postalbumin seems to correspond to human  $\alpha_f$ -globulin.

Antigen LA purified from rat amniotic fluid by Stanislawski-Birencwajg (27) has a smaller sedimentation coefficient

cient (2.9 S), a larger extinction coefficient, quite different amino acid composition, and euglobulin nature. In spite of the discrepancy, antigen LA, as well as human  $\alpha$ -globulin, is one of the most predominant fetal serum  $\alpha$ -globulins and disappears with an advance of age. In addition, both appear specifically with liver cancer. It is difficult to assess whether or not antigen LA belongs to the same protein species. It would seem, however, possible that antigen LA in serum is identical to postalbumin but somewhat different from that in amniotic fluid.

$\alpha$ -Globulin appearing in the serum of hepatoma patients has been reported to be identical to fetal  $\alpha$ -globulin by an immunological method.  $\alpha$ -Globulin was prepared from both sera and characterized in the present study. However, no essential difference could be detected between these 2 preparations by physicochemical and chemical criteria.

An immunochemical method was used for purification in this study. This method was simple, and the yield was excellent. Owing to the specificity of antigen-antibody reaction,  $\alpha$ -globulin was prepared in a high degree of purity from serum which is a complex mixture of various proteins containing only a small amount of this globulin. Purified preparations showed electrophoretic mobility, antigenicity in the double diffusion test, and gel filtration behavior identical to those of  $\alpha$ -globulin in serum. This protein seems stable at the low pH used in this method.

The amino acid analyses reported in the present study indicate the presence of the usual amino acids found in other serum proteins. The content of the peptide portion, estimated from the recovery of amino acids analyzed and the content of nonnitrogenous sugars, is approximately 97%. Nonnitrogenous sugars are estimated to be present about 3% in the purified material but the possibility of contamination of this specimen with glucose derivatives derived from Sephadex was not completely excluded. Traces of hexosamine could be detected during amino acid analyses. The structure of the carbohydrate components of this protein is under investigation.

## ACKNOWLEDGMENTS

I extend my appreciation to Professor Hidematsu Hirai for his excellent instruction and for his council. I also thank Dr. Howard B. Hamilton, Atomic Bomb Casualty Commission, Hiroshima, Japan, for reading this manuscript and Dr. Kunihiro Kobayashi, my colleague, for his valuable discussion.

## REFERENCES

1. Abelev, G. I. Production of Embryonal Serum  $\alpha$ -Globulin by Hepatomas: Review of Experimental and Clinical Data. *Cancer Res.*, **28**: 1344-1350, 1968.
2. Abelev, G. I., Perova, S. D., Khramkova, N. I., Postnikova, Z. A., and Irlin, I. S. Production of Embryonal  $\alpha$ -Globulin by Transplantable Mouse Hepatomas. *Transplantation*, **1**: 174-180, 1963.
3. Albert, M. E., Uriel, J., and de Nechaud, B. Alpha<sub>1</sub> Fetoglobulin in the Diagnosis of Human Hepatoma. *New Engl. J. Med.*, **278**: 984-986, 1968.
4. Andreoli, M., and Robbins, J. Serum Proteins and Thyroxine-Protein Interaction in Early Human Fetuses. *J. Clin. Invest.*, **41**: 1070-1077, 1962.
5. Bencze, W. L., and Schmid, K. Determination of Tyrosine and Tryptophan in Proteins. *Anal. Chem.*, **29**: 1193-1196, 1957.
6. Benson, J. V., Jr., and Patterson, J. A. Accelerated Automatic Chromatographic Analysis of Amino Acids on a Spherical Resin. *Anal. Chem.*, **37**: 1108-1110, 1965.
7. Bergstrand, C. G., and Czar, B. Demonstration of a New Protein Fraction in Serum from the Human Fetus. *Scand. J. Clin. Lab. Invest.*, **8**: 174, 1956.
8. Bergstrand, C. G., and Czar, B. Paper Electrophoretic Study of Human Fetal Serum Protein with Demonstration of a New Protein Fraction. *Scand. J. Clin. Lab. Invest.*, **9**: 277-286, 1957.
9. Davis, B. J. Disc Electrophoresis-II. Method and Application to Human Serum Proteins. *Ann. N. Y. Acad. Sci.*, **121**: 404-427, 1964.
10. Francois, C., Marshall, R. D., and Neuberger, A. Carbohydrates in Protein. 4. The Determination of Mannose in Hen's Egg Albumin by Radioisotope Dilution. *Biochem. J.*, **83**: 335-341, 1962.
11. Gitlin, D., and Boesman, M. Serum  $\alpha$ -Fetoprotein, Albumin and  $\gamma$ -Globulin in the Human Conceptus. *J. Clin. Invest.*, **45**: 1826-1838, 1966.
12. Gitlin, D., and Boesman, M. Fetus-specific Serum Proteins in Several Mammals and Their Relation to Human  $\alpha$ -Fetoprotein. *Comp. Biochem. Physiol.*, **21**: 327-336, 1967.
13. Grabar, P., Stanislawski-Birencwajg, M., Oisgold, S., and Uriel, J. Immunochemical and Enzymatic Studies on Chemically Induced Rat Liver Tumors. In: R. J. C. Harris (ed.), *Specific Tumor Antigens*, UICC Monograph Series, Vol. 2, pp. 20-31. Copenhagen: Einar Munksgaard, 1967.
14. Grabar, P., and Williams, C. A. Méthode Permettant l'Étude Conjuguée des Propriétés Electrophorétiques et Immuno-chimiques d'un Mélange de Protéines. Application au Sérum Sanguin. *Biochim. Biophys. Acta*, **10**: 193-194, 1953.
15. Hull, E. W., Carbone, P. P., Gitlin, D., O'Gara, R. W., and Kelly, M. G.  $\alpha$ -Fetoprotein in Monkeys with Hepatoma. *J. Natl. Cancer Inst.*, **42**: 1035-1044, 1969.
16. Kawahara, K. Evaluation of Diffusion Coefficient of Proteins from Sedimentation Boundary Curves. *Biochemistry*, **8**: 2551-2557, 1969.
17. Kirsh, J. A. W., Wise, R. W., and Oliver, I. T. Postalbumin, a Foetal-specific Rat Plasma Protein. Purification, Physicochemical and Immunological Studies. *Biochem. J.*, **102**: 763-766, 1967.
18. Kithier, K., Houštěk, J., Masopust, J., and Rádl, J. Occurrence of a Specific Foetal Protein in a Primary Liver Carcinoma. *Nature*, **212**: 414, 1966.
19. Mancini, G., Carbonara, A. O., and Heremans, J. F. Immunochemical Quantitation of Antigens by Single Radial Immunodiffusion. *Immunochemistry*, **2**: 235-254, 1965.
20. McMeekin, T. L., Groves, M. L., and Hipp, N. J. Apparent Specific Volume of  $\alpha$ -Casein and  $\beta$ -Casein and the Relationship of Specific Volume to Amino Acid Composition. *J. Am. Chem. Soc.*, **71**: 3298-3300, 1949.
21. Ouchterlony, O. Antigen-Antibody Reaction in Gels. *Arkiv Kemi Mineral. Geol.*, **26B**: 14, 1948.
22. Pedersen, K. O. Fetuin, a New Globulin Isolated from Serum. *Nature*, **154**: 575, 1944.
23. Purves, L. R., MacNab, M., Geddes, E. W., and Bersohn, I. Serum  $\alpha$ -Fetoprotein and Primary Hepatic Cancer. *Lancet*, **1**: 921-922, 1968.
24. Schöniger, W. Die Mikroanalytische Schnellbestimmung von Halogenen und Schwefel in Organischen Verbindungen. *Mikrochim. Acta.*, 869-876, 1956.
25. Spiro, R. G. Studies on Fetuin, a Glycoprotein of Fetal Serum. I. Isolation, Chemical Composition and Physicochemical Properties. *J. Biol. Chem.*, **235**: 2860-2869, 1960.

26. Spiro, M. J., and Spiro, R. G. Composition of the Peptide Portion of Fetuin. *J. Biol. Chem.*, 237: 1507-1510, 1962.
27. Stanislawski-Birencwajg, M. Specific Antigens of Rat Embryonic Serum. *Cancer Res.*, 27: 1982-1989, 1967.
28. Svedberg, T. The Ultra-Centrifuge and the Study of High-Molecular Compounds. *Nature*, 139: 1051-1062, 1937.
29. Tatarinov, Y. S. New Data on the Embryo-specific Antigenic Components of Human Blood Serum. *Vopr. Med. Khim.*, 10: 584-589, 1964. (*Federation Proc.*, 24: T916-T918, 1965.)
30. Tatarinov, Y. S. Content of Embryo-specific  $\alpha$ -Globulin in Fetal and Neonatal Sera and Sera from Adult Humans with Primary Carcinoma of the Liver. *Vopr. Med. Khim.*, 11: 20-24, 1965 (*Federation Proc.*, 25: T344-T346, 1966.)
31. Uriel, J., de Nechaud, B., Stanislawski-Birencwajg, M., Masseyeff R., Leblanc, L., Quenum, C., Loisillier, F., and Grabar, P. L. Diagnostic du Cancer Primaire du Foie par des Méthode Immunologiques. *Presse Med.*, 76: 1415-1417, 1968.
32. Wise, R., Ballard, F., and Ezikiel, E. Developmental Changes in the Plasma Protein Pattern of the Rat. *Comp. Biochem. Physiol.* 9: 23-30, 1963.

---

Fig. 1. Immunelectrophoretic pattern of fetal (F) and normal adult (A) serum against unabsorbed (aF) and absorbed (ab. aF) rabbit antiserum to fetal serum. Anode is to the right. Note reaction for  $\alpha$ <sub>F</sub>-globulin between fetal serum and absorbed antiserum which is absent between normal adult serum and absorbed antiserum.

Fig. 2. Double immunodiffusion pattern of fetal serum (F), normal adult serum (A), hepatoma patient serum (H), and  $\alpha$ <sub>F</sub>-globulin prepared from a hepatoma patient (Ha<sub>F</sub>) and from fetuses (Fa<sub>F</sub>) against horse antiserum (aa<sub>F</sub>) to  $\alpha$ <sub>F</sub>-globulin prepared by immunization with antigen-antibody precipitate formed between the absorbed rabbit antiserum and a hepatoma patient serum. Note the fused precipitation lines indicating complete antigenic identity and absence of reaction between normal adult serum and the antiserum.

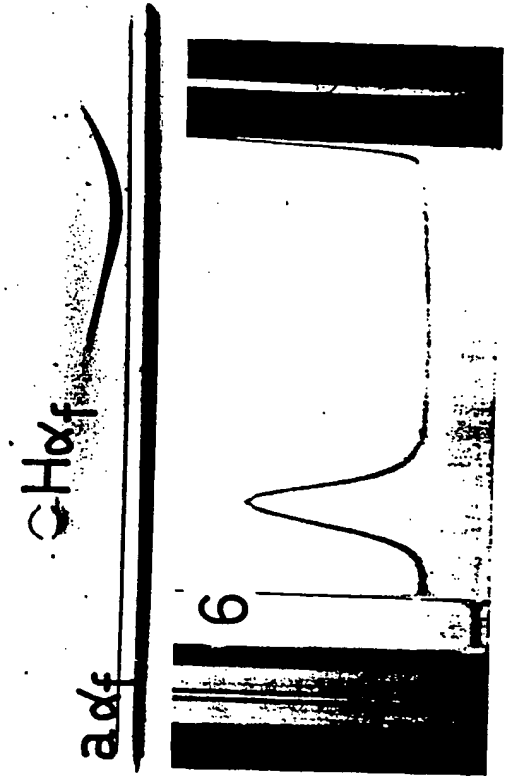
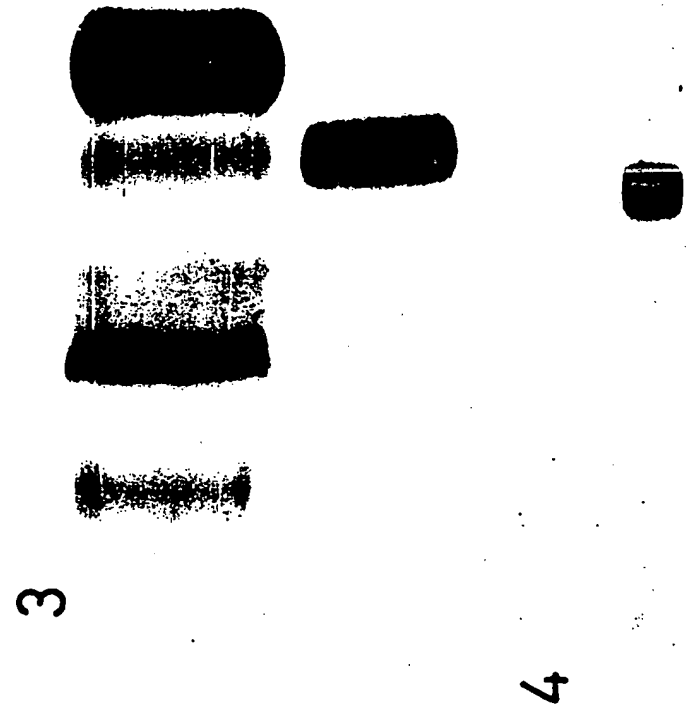
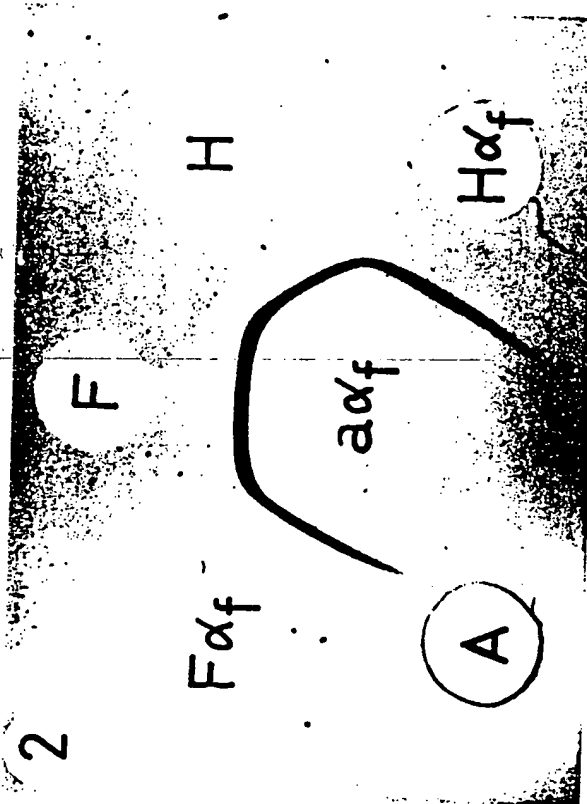
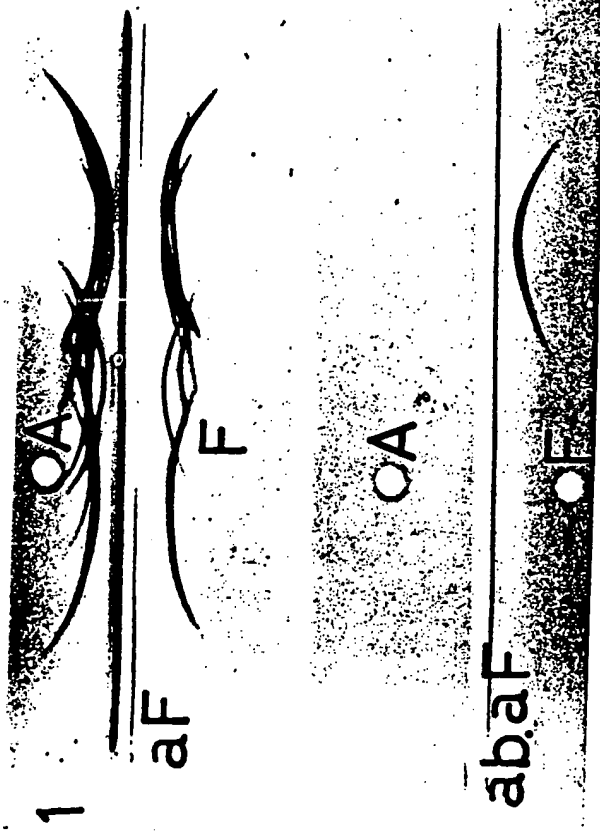
Fig. 3. Cellulose acetate membrane electrophoretic pattern of hepatoma patient serum (above) and  $\alpha$ <sub>F</sub>-globulin from a hepatoma patient (below).  $\alpha$ <sub>F</sub>-Globulin migrated as a single band between albumin and  $\alpha$ <sub>1</sub>-globulin. Buffer, 0.05 M barbital, pH 8.6; potential gradient, 20 V/cm; time, 45 min. Anode is to the right.

Fig. 4. Disc electrophoretic pattern of  $\alpha$ <sub>F</sub>-globulin from a hepatoma patient.  $\alpha$ <sub>F</sub>-Globulin was homogeneous, migrating as a single band. Anode is to the right.

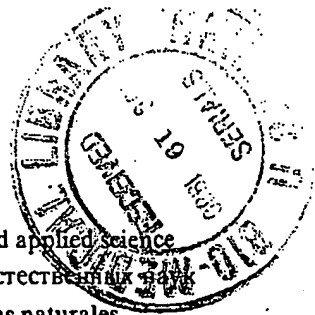
Fig. 5. Immunelectrophoretic pattern of  $\alpha$ <sub>F</sub>-globulin from a hepatoma patient. (Ha<sub>F</sub>) and hepatoma patient serum (H) against horse antisera to  $\alpha$ <sub>F</sub>-globulin (aa<sub>F</sub>) and to normal human serum (aA). Note that no reaction was observed between  $\alpha$ <sub>F</sub>-globulin and antisera to normal human serum. Anode is to the right.

Fig. 6. Ultracentrifugal pattern of  $\alpha$ <sub>F</sub>-globulin from a hepatoma patient serum.  $\alpha$ <sub>F</sub>-Globulin was dissolved at 0.8% in 0.15 M NaCl solution. Ultracentrifugation was carried out at 56,100 rpm for 48 min. A single symmetric peak was observed.

Essentially the same results were obtained if  $\alpha$ <sub>F</sub>-globulin from fetuses were analyzed.



4



Revue mensuelle des sciences pures et appliquées · Monthly journal of pure and applied science  
 Monatsschrift für die Gebiete der Naturwissenschaft · Ежемесячный журнал естественных наук  
 Rivista mensile di scienze pure e applicate · Revista mensual de las ciencias naturales

Editores: H. Aebi, Bern · D. Arigoni, Zürich · P. S. Chen, Zürich · A. Pletscher, Basel · S. Weidmann, Bern · R. Weil, Genève

Redactor: H. Mislin, Basel · Carona

Birkhäuser Verlag, Basel · Boston · Stuttgart

## Table des matières - Inhaltsverzeichnis - Sommario - Contents - Содержание - Indice

### Generalia

#### The carbon dioxide problem. An interdisciplinary survey

1. Introduction  
W. Bach ..... 767
2. The carbon cycle: sources and sinks of atmospheric CO<sub>2</sub>  
G. H. Kohlmaier, U. Fischbach, G. Kratz, H. Bröhl and W. Schunck ..... 769
3. The effect of the atmosphere-biosphere exchange on the global carbon cycle  
U. Hampicke ..... 776
4. Past and future emission of CO<sub>2</sub>  
R. M. Rotty ..... 781
5. Prediction of future CO<sub>2</sub> concentrations in the atmosphere  
U. Siegenthaler and H. Oeschger ..... 783
6. Physiological aspects of increased CO<sub>2</sub> concentration  
H. van Keulen, H. H. van Laar, W. Louwerse and J. Goudriaan ..... 786
7. The impact of different energy options on atmospheric CO<sub>2</sub> levels  
F. Niehaus ..... 792
8. Climatic effects of increasing atmospheric CO<sub>2</sub> levels  
W. Bach ..... 796
9. Measures of CO<sub>2</sub> control  
C. Marchetti ..... 806
10. Conclusions  
H. Oeschger ..... 808

### Specialia

#### Mathematica, Physica

- The critical rotation of gravitating degenerate cylinders  
Z. F. Seidov ..... 813

#### Chimica, Biochimica

- Furodendin, a C<sub>21</sub> degraded terpene from the sponge *Phyllospongia dendyi*  
R. Kaslauskas, P. T. Murphy and R. J. Wells ..... 814
- Synthesis of koeniginine  
R. B. Sharma, R. Seth-Verma and R. S. Kapil ..... 815
- Two 2-dimensional electrophoreses: A combination of crossed immunoelectrophoresis and dodecyl sulfate polyacrylamide gel electrophoresis for protein analysis  
T. Yamamoto and Y. Tanada ..... 816
- Monamine oxidase inhibitors and histamine metabolism  
M. Strolin Benedetti, J. F. Ancher and N. Sontag ..... 818
- The effect of pH and methylation on the interaction of deoxycholate with rat liver alcohol dehydrogenase  
M. Simonetta and G. M. Hanozet ..... 820
- Co-polymerization of dopa and cysteinyl-dopa in melanogenesis in vitro  
S. Ito, E. Novellino, F. Chioccare, G. Misuraca and G. Prota ..... 822

- The response of cyclic 3',5'-AMP and cyclic 3',5'-GMP phosphodiesterases to experimental diabetes  
B. Perez de Gracia, A. R. Rhoads and W. L. West ..... 824
- A comparison of 3 reagents in converting thiamine to thiochrome in the presence of plant extracts and polyphenols  
K. Ratanaulochai, S. Pikulkarntaler and B. Pantijan ..... 825

### Biologica

- Karyotypes of several frogs from Korea, Taiwan and the Philippines  
M. Kuramoto ..... 826
- Effect of ethanol and isopropanol on the activity of alcohol dehydrogenase, viability and life-span in *Drosophila melanogaster* and *Drosophila funebris*  
L. Vilageliu Argués and R. Gonzalez Duarte ..... 828
- Spectral sensitivity in a fresh-water Gastrotich (*Lepidodermella squamatum* Dujardin)  
M. Balsamo ..... 830
- Identification of intracellular particles of pesticides in ciliate protozoa by Raman microprobe  
D. Dive, J. M. Devynck, G. Leroy, M. N. Fourmaux and Y. Moschetto ..... 832
- Neurosecretory system and storage of paraldehyde fuchsin positive neurosecretory material in the dorsal aorta in an earwig, *Anisolabis annulipes* Lucas (Dermaptera: Labiduridae)  
Y. N. Singh and R. Narain ..... 834
- The effect of RNA synthesis inhibitors on prenucleolar bodies  
Gloria Morcillo and C. de la Torre ..... 836
- Satellite DNA sequences and reproductive isolation in the *Drosophila willistoni* group  
G. Corneo, L. Ceccherini Nelli, Donatella Meazza and F. J. Ayala ..... 837
- Absorption of glycine and proline from the small intestine of rats infected with *Elmeria nieschulzi*  
S. J. Ball, Christine E. Heading and B. Tranter ..... 839

### Physiologica

- Effects of altered thyroid state on the inhibition produced by locus coeruleus in Purkinje cells in the rat  
Dolores Fuenmayor and J. A. Gonzalez-Vegas ..... 841
- Microsphere measurement of myocardial capillary bed in hypoxic rats  
A. J. Stere, N. W. Brister and A. Anthony ..... 842
- Catalase activity in electrically stimulated muscle  
R. R. Jenkins and D. Newsham ..... 843
- Effects of inhibition and stimulation of Na<sup>+</sup>-K<sup>+</sup> active transport on the resting membrane input conductance of the guinea-pig ventricle  
T. M. Nosek ..... 844
- Dietary utilization of aliphatic alcohols by *Drosophila*  
J. Van Herreweghe, J. R. David and R. Grantham ..... 846

vide paginam aversam

the damage of the periventricular structures. An additional advantage of this method is the simplicity and unexpensiveness of the technical tools as compared to the methods using pressure transducers and recording devices<sup>4,9</sup>.

- 1 This work was done under a contract with the Association of Finnish Life Insurance Companies.
- 2 F.U. Gerdes, G. Klein, M. Nadjmi and G. Schaltenbrand, *J. Neurol.* 210, 183 (1975).

- 3 K.I. Melville and H.E. Shister, *Am. Heart J.* 53, 425 (1957).
- 4 L. Eriksson, *Acta physiol. scand.*, suppl. 444, 8 (1976).
- 5 H. Schmitt and H. Schmitt, *Eur. J. Pharmac.* 6, 8 (1969).
- 6 V.S. Vassiliev and A.O. Ramos, *Pharmacology* 13, 97 (1975).
- 7 E.K. Walls and T.B. Wishart, *Physiol. Behav.* 19, 171 (1977).
- 8 A. Hernandez, S. Ruiz and C. Pacile, *Experientia* 31, 1475 (1975).
- 9 G.A. Goodrich, B. Greehey, T.B. Miller and J.R. Pappenheimer, *J. appl. Physiol.* 26, 137 (1969).

NOT REPRODUCIBLE WITHOUT PERMISSION  
OF THE PUBLISHERS (TITLE 17 U.S. CODE)

## Immunogenicity of agarose-immobilized immune complexes<sup>1</sup>

P.J. Higgins

Laboratory of Cell Biochemistry, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York (N.Y. 10021, USA), 8 October 1979

**Summary.** A novel method is described for production of heterologous antisera to a specific tumor-associated murine antigen by immunization with agarose-trapped immune complexes.

Preparation of high titer heterologous antisera to specific tumor-associated antigens is complicated by the use of crude tissue extracts as immunogen in which the relative concentration of the antigen is quite low. Where the tumor-associated antigen is present in moderate quantities, reasonable quality antisera can be obtained by immunization with unfractionated tissue extracts and appropriate serum absorptions. Such antisera have been used to precipitate antigen from solution and the immune complexes, in turn, employed as immunogens to elicit the formation of a more highly specific antiserum<sup>2</sup>. The limiting factors, however, of sufficiently absorbed precipitating antibody, low concentrations of specific antigen in crude tissue extracts and difficulties in the efficient processing of minute precipitates combine to make effective immunization with solution-precipitated immune complexes a prodigious task.

A simple method for the preparation of high titer antisera to a specific tumor-associated antigen, murine  $\gamma$ -FA<sup>3-5</sup>, is described. This procedure should be applicable to studies of diverse tissue antigens.

**Methods and results.** Immunization of rabbits with saline extracts of a mouse fibrosarcoma, antiserum absorption and subsequent identification of an antigen common to tumor, fetal and adult splenic tissue, termed  $\gamma$ -FA, has been described<sup>3-5</sup>. Radial immunodiffusion plates<sup>6</sup> contained 0.1 ml of absorbed anti- $\gamma$ -FA serum and 2.5 ml of 1% agarose (w/v) in Beckman B-2 buffer, pH 8.6. Antigen wells (3.7 mm in diameter) were cut into the agarose gel, filled with 7  $\mu$ l of the 10,000 $\times$ g supernatant fraction of a

10<sup>-3</sup> M Tris, pH 7.5, homogenate of normal adult mouse spleen and the plates incubated at 37°C for 72 h to allow for precipitin ring formation. The agarose slabs were then dialyzed with stirring against daily 200-ml changes of phosphate-buffered saline (PBS) for 3 weeks at 4°C in order to remove unbound protein. The use of a 1% agarose gel facilitated this removal while antigen-antibody complexes remained trapped within the gel matrix. After dialysis, sections of gel containing precipitin rings were cut out, passed several times through an 18-gauge needle and frozen at -20°C in twice the volume of PBS. On day 1, 14 and 21, 1 ml of agarose-immobilized immune complexes was emulsified in 1 ml of complete Freund's adjuvant and inoculated s.c. into a New Zealand white rabbit (on day 21 incomplete adjuvant was substituted for complete adjuvant). 22 days later the rabbit was bled and 1-ml aliquots of the antiserum inoculated i.p. into each of several adult C57 mice for in vivo absorption. After 24 h, the mice were bled and the antibody activity of the absorbed antiserum compared with that of the original anti- $\gamma$ -FA serum. Clearly, the antiserum to  $\gamma$ -FA-anti- $\gamma$ -FA immune complexes possessed all the precipitin specificity of the original anti- $\gamma$ -FA serum but at a much higher titer (table) and, unlike the original antiserum, yielded a positive indirect immunofluorescence test<sup>4</sup> on methanol-fixed rat hepatoma cells (figure). This antigen was previously thought to be synthesized only by in vivo propagated tumor cells<sup>4</sup> and the present data, therefore, provide the 1st direct evidence for production of  $\gamma$ -FA by transformed cells. Moreover, reten-



Fig. 1. Indirect immunofluorescence test of in vitro propagated rat tumor cells isolated from a transplanted hepatoma<sup>7</sup>. A Original anti- $\gamma$ -FA serum (1:20); B anti- $\gamma$ -FA immune complexes (1:60). UV light microscopy, BG12-53/44 filters, Zeiss Photomicroscope.



Table 1. Comparative precipitin tests<sup>a</sup>

Test antigen	Original anti- $\gamma$ -FA (undiluted)	Anti- $\gamma$ -FA immune complexes (1:30) <sup>b</sup>
Meth A serum <sup>c</sup>	+	+++
Meth A tumor <sup>d</sup>	+	++++
Normal serum <sup>e</sup>	—	—
Adult spleen <sup>f</sup>	+	++++
Viscera <sup>g</sup>	—	—

<sup>a</sup> Hyland Immuno-Plates, pattern 'D'. Preliminary experiments indicated the precipitin line which formed upon interaction of the original anti- $\gamma$ -FA serum with a saline extract of adult mouse spleen and that which formed by interaction of anti- $\gamma$ -FA immune complexes with the same splenic tissue extract was one of identity.

<sup>b</sup> Calculated dilution after in vivo absorption. <sup>c,d</sup> Serum and saline extract of tumor tissue obtained from a mouse bearing a transplanted 3-methylcholanthrene-induced fibrosarcoma. <sup>e,f</sup> Obtained from normal adult mice. <sup>g</sup> Individual saline extracts of adult mouse liver, kidney, brain, heart, lung, testes and small intestine pooled from several mice.

tion of antibody activity after in vivo absorption in mice (which have  $\gamma$ -FA-positive spleens) suggests that  $\gamma$ -FA is not a cell surface antigen.

Preparation of a high titer anti- $\gamma$ -FA serum by immunization with agarose-trapped immune complexes will facilitate future in vitro studies of various aspects of cellular transformation. In addition, the method described should be adaptable to diverse tissue antigen systems.

1 Supported in part by a Biomedical Research Support Grant and Grant ROI-CA-20019-04 from the National Cancer Institute.

2 S. Nishi, *Cancer Res.* 30, 2507 (1970).

3 C. Tong, E.H. Stonehill, P.J. Higgins and A. Bendich, *Eur. J. Cancer* 14, 147 (1978).

4 P.J. Higgins, C. Tong, E. Borenfreund and A. Bendich, *Eur. J. Cancer* 15, 423 (1979).

5 P.J. Higgins, C. Tong, E. Borenfreund, R.S. Okin and A. Bendich, *Br. J. Cancer* 40, 253 (1979).

6 G. Mancini, A.O. Carbonara and J.F. Heremans, *Immunochimistry* 2, 235 (1965).

7 E. Borenfreund and P.J. Higgins, *Ann. N.Y. Acad. Sci.*, in press (1980).

## CONGRESSUS

### Great Britain

#### 4th meeting of European neuroscience association

Brighton, 13-20 September 1980

Plenary lectures will be presented by H. Barlow, M. Burger, A. Burgin, E. Zaidel, S. Thesleff, P. Andersen, D. Ploog and L. Heimer.

For further information, please write to the secretary of the meeting, 142 Oxford Road, Cowley, Oxford OX4 2DZ, England.

### Switzerland

#### 12th FEMS symposium "Microbial degradation of xenobiotics and recalcitrant compounds"

Zürich, 15-17 September 1980

Main lectures on "Xenobiotics in the environment", "Cellular and subcellular aspects of biodegradation research" and "Biodegradation of specific compounds". For information contact Prof. Th. Leisinger, Mikrobiologisches Institut ETH, ETH-Zentrum, CH-8092 Zürich.

### Instructions to authors

Experientia is published on the 15th of every month and can be obtained in any country through booksellers or from the publishers. All communications to the editors should be addressed to the publishers. All manuscripts for publication in a given number must be in the hands of the editors 3 months before publication.

Articles of general scientific interest, of interdisciplinary character: briefly stated and hitherto unpublished original reports of sufficient novelty value.

Experiments Papers in which animal experiments have been conducted without using the appropriate anaesthesia will not be accepted.

Manuscripts (including all figures and tables) should be submitted in duplicate.

Text should not exceed 2-3 typewritten pages (50-60 lines). 1-2 relevant figures or tables. English summary of maximum 4 lines. Abbreviations should be properly explained. References should be numbered consecutively and be presented on a separate page. Name and address have to be placed directly under the title. Linguistically inadequate manuscripts will be returned. Manuscripts in languages other than English should be supplemented by an English translation of the title. Footnotes should be avoided.

Figures Illustrations should be separate from the text, with the author's name on the back in soft pencil. The desired labelling should be shown on a second set of figures, which will be used as a model for

inscriptions. Drawings for reproductions should be on good paper in Indian ink. photographs should be supplied as glossy positive prints. The illustrations should be at least one and a half times as large as the definitive size desired. Over-large figures can be easily damaged in the mail. Captions should be selfexplanatory, without reference to the text.

Tables should be provided with a title and with selfexplanatory captions.

Headings In submitting their manuscript to Experientia, authors are requested to indicate one of the headings mentioned below, under which they would wish to place their short communication:

1 Mathematics and Physics; 2. Cosmology, Astronautics, Cosmonautics; 3. Mineralogy, Geophysics, Oceanography; 4. Inorganic and Physical Chemistry; 5. Organic Chemistry; 6. Biophysics; 7. Molecular Biology, Cellular Biology; 8. Genetics; 9. Botany; 10. Zoology; 11. Ecology; 12. Biochemistry (analytic and synthetic); 13. Biochemistry (Enzymes, Metabolism); 14. Physiology; 15. Neurology; 16. Pharmacology, Toxicology, Pathology; 17. Experimental Gerontology; 18. Anatomy, Histology, Cytology, Histochemistry; 19. Embryology; 20. Endocrinology; 21. Circulation, Cardiology, Angiology; 22. Nutrition, Gastroenterology; 23. Hematology, Serology; 24. Immunology, Allergy; 25. Microbiology, Parasitology, Chemical Therapeutics; 26. Oncology, Carcinology, Cytostatics; 27. Radiology.

Reprints The authors receive 50 reprints, without cover, free of charge. Price-list for further reprints is available.

# immunological methods

Editors:

E.J. HOLBOROW  
V. NUSSENZWEIG



Volume 64, Nos. 1-2, 1983

JIM 02818

NOTICE: THIS MATERIAL MAY BE PROTECTED  
BY COPYRIGHT LAW (TITLE 17 U.S. CODE)

# The Use of Conventional Antisera in the Production of Specific Monoclonal Antibodies

Kendra B. Eager<sup>1</sup> and Roger H. Kennett

*Department of Human Genetics, University of Pennsylvania, School of Medicine, Philadelphia,  
PA 19104, U.S.A.*

(Received 5 May 1983, accepted 27 May 1983)

Monoclonal antibodies specific for human  $\alpha$ -2-macroglobulin ( $\alpha_2$ M), a plasma glycoprotein, have been produced using a novel immunization method. Commercially available antisera to human  $\alpha_2$ M was used to precipitate the antigen from whole serum. Immunization of animals with this immunoprecipitate resulted in the production of hybridomas with a specificity for human  $\alpha$ -2-macroglobulin as confirmed by immunoprecipitation.

---

Key words: *antisera - monoclonal antibody -  $\alpha$ -2-macroglobulin - immunization with immune complexes*

---

## Introduction

During the past few years, monoclonal antibodies have become a tool for the detection of cell surface molecules and for the fine structure analysis of macromolecular antigens. As in the initial report of monoclonal antibody production (Kohler and Milstein, 1975), antigen preparations have often consisted of a complex mixture of molecules. Although monoclonal antibodies have the advantage of usually reacting with only one of the components of a heterogeneous mixture, it is often difficult to determine which of the many antibodies produced is actually against the molecule of interest. There is, therefore, a distinct advantage in cases where the immunogen and the antigens used for screening are as pure a preparation as possible. In cases where a conventional antisera against the antigen of interest is available, immunization with the antigen precipitated with this serum can be an effective way to produce monoclonal antibodies.

We have chosen to use this method to produce specific monoclonal antibodies against human  $\alpha$ -2-macroglobulin ( $\alpha_2$ M).  $\alpha$ -2-macroglobulin, a glycoprotein pro-

---

<sup>1</sup> Address correspondence to: Dr. K.B. Eager, The Wistar Institute, 36th and Spruce Streets, Philadelphia, PA 19104, U.S.A.

tease inhibitor in human plasma, is present in quantities ranging from 1.5 to 2 mg/ml depending upon the age and sex of the individual (Laurell and Jeppsson, 1975). Using goat anti-human  $\alpha_2$ -macroglobulin serum, human  $\alpha_2$ M was precipitated from serum and injected into animals. Hybridomas produced by the fusion of Sp2/0-Ag14 and sensitized spleen cells were screened for their specificity of reaction by an adaptation of the enzyme-linked immunosorbent assay reported by Engvall and Perlmann (1971).

## Materials and Methods

### *Parental cell line*

Sp2/0-Ag14 (Shulman et al., 1978), a non-immunoglobulin producing mouse plasmacytoma cell line, was maintained in Dulbecco's Modified Eagle's Medium with high glucose (DMEM-Hg) supplemented with 10% calf serum and glutamine.

### *Preparation of protein for immunizations*

$\alpha_2$ -macroglobulin ( $\alpha_2$ M) was isolated from human serum using anti-human  $\alpha_2$ M (Cappel Laboratories, Cochranville, PA). Both serum and antiserum were stored at  $-70^\circ\text{C}$ . The antibody-antigen complex obtained from the mixture of the antiserum and serum was prepared in double diffusion plates (Kallestad, Chaska, MN). After extensive washing in phosphate buffered saline (PBS), pH 7.2, to remove other proteins from the gel, the precipitin bands were excised from the surrounding gel.

### *Immunization procedure*

The precipitin bands were homogenized, resuspended in PBS and injected into female BALB/c mice (Ace Animal Suppliers). Material from six bands (i.e. the amount of  $\alpha_2$ M precipitated from 15  $\mu\text{l}$  of serum) were injected into mice at weekly intervals for 3–8 weeks. The route of immunization alternated between intraperitoneal and subcutaneous injections. For the 3 days prior to the fusion, the mice were given daily intravenous injections of the immunoprecipitate containing twice the amount of protein given on previous occasions. The same procedure was followed for immunization of rats although the amount of antigen in each immunization was doubled. On the day of the fusion, the spleen was removed aseptically and blood was collected for titration of anti- $\alpha_2$ -macroglobulin antibodies. This serum was used as a control serum in ELISA assays.

### *Fusion*

The fusion, cloning, and maintenance of the hybrid cells were performed according to the method reported by Kennett et al. (1978).

### *ELISA screening assay*

Screening for those hybridomas producing immunoglobulin specific for human  $\alpha_2$ M was modified from a technique described by Kennett (1980). The source of the human  $\alpha_2$ M was an immunoprecipitate resulting from the reaction of human serum

(containing 0.1 mg soybean trypsin inhibitor (Sigma) and 500 KIU (kallikrein inactivator units) of Trasylol (aprotinin) (FBA Pharmaceuticals) per milliliter) with the goat antiserum. The precipitated protein was washed three times in PBS, diluted in 0.05 M sodium carbonate buffer, pH 9.8, and distributed to the wells of a 96-well protein binding polystyrene plate (Microelisa plates, Dynatech Laboratories, Inc., Alexandria, VA). Control plates were prepared using goat anti-human  $\alpha_2$ M alone. After the plates had been incubated at 4°C for 18 h, they were washed twice by immersion and flicking with a 0.05% Tween 80 (Sigma) solution in PBS. Monoclonal antibody was added and incubated for 2 h at room temperature. Following several washings in Tween 80/PBS, the peroxidase conjugated goat anti-mouse immunoglobulin (Cappel Laboratories), absorbed with an equal volume of purified human immunoglobulin (Cappel Laboratories) to remove cross-reacting antibodies and diluted in Tween 80/PBS containing 0.05 M EDTA and 2% additional NaCl, was added to the wells. The plates were incubated for two additional hours at room temperature and washed 5–7 times in Tween 80/PBS. After the final wash, 150  $\mu$ l of solution of 5.5 mM *o*-phenylene diamine, 0.012 M hydrogen peroxide, dissolved in 0.1 M citrate buffer, pH 4.5, was added to each well. The enzyme reaction liberating a colored product was allowed to proceed for 20–30 min before stopping the reaction with 0.1 M sodium fluoride. The color was read quantitatively on a Multiscan spectrophotometer (Flow Laboratories) at 450 nm.

#### *Determination of immunoglobulin class*

Class of immunoglobulin was determined by precipitation using class specific antisera (Miles Laboratories, Elkhart, IN) in double diffusion gels.

#### *Ammonium sulfate precipitation of immunoglobulin*

Hybridomas were grown in mass culture in DMEM-Hg supplemented with 5% calf serum. Cells were grown to mid-log phase and maintained until 50% of the cells were unhealthy and supernatant harvested. Equal volumes of saturated ammonium sulfate solution and culture supernatant were mixed. The precipitate was spun at 48,000  $\times$  g, dialyzed against PBS, and frozen at  $-70^\circ\text{C}$  (Jonak, 1980).

#### *Competition assay*

Actively growing cultures of hybridomas were labeled with L-[ $^{35}\text{S}$ ]methionine (Amersham U.K.) after being washed in methionine-deficient RPMI 1640 (Grand Island Biological Co., NY) supplemented with 30 mg/ml glutamine (Sigma) and 5% fetal calf serum. A volume of 250  $\mu$ l of [ $^{35}\text{S}$ ]methionine was added to  $10^7$  cells and incubated for 18 h in a 5%  $\text{CO}_2$  incubator. Twenty minutes before harvesting the supernatant, 0.1 ml of 10 mM methionine was added.

After overnight incubation at 4°C, 2.5  $\mu$ g of  $\alpha_2$ M immunoprecipitate dissolved in 0.2 M phosphate buffer, pH 8.0, was attached per well of 96-well polyvinylchloride (PVC) plates (Dynatech). The PVC plates were blocked for 1 h with incubation buffer containing 0.1 M phosphate buffer, 0.15 M NaCl, 0.02%  $\text{NaN}_3$ , 0.25% BSA and 2.0% calf serum. Unlabeled antibody in the form of dense culture supernatant was added (50  $\mu$ l/well) and incubated for 2 h. Then 50  $\mu$ l of biosynthetically labeled

[ $^{35}\text{S}$ ]methionine monoclonal antibody was added and incubated for an additional 2 h. The wells were washed, dried and counted.

#### *Monoclonal antibody immunoprecipitation*

Formalin-fixed *Staphylococcus aureus* (Calbiochem) coated with goat anti-mouse immunoglobulin (Cappel) was washed and incubated with monoclonal antibodies according to the method of Lampson (1980). After the incubation with antibody, antigen in the form of whole human serum containing soybean trypsin inhibitor and Trasylol in concentrations mentioned above was incubated for 18 h with *S. aureus*-antibody complex. After washing, the antibody-antigen complex was eluted from the *S. aureus* by boiling for 2 min in sample buffer containing 0.125 M Tris (Sigma), 2.2 M glycerol (Mallinckrodt), 0.2% Coomassie Brilliant Blue R250 (BioRad), 0.16 M sodium dodecyl sulfate (SDS)(BioRad), and 10%  $\beta$ -mercaptoethanol (Sigma). The samples were spun in a Beckman ultrafuge and applied to a 5% acrylamide-0.13% bis-acrylamide gel with 0.1% SDS and run at constant current. The gel was subsequently stained with 0.2% Coomassie Brilliant Blue R250 in methanol-water-glacial acetic acid (5:5:1), destained and dried on a gel slab dryer (BioRad).

#### *Starch gel electrophoresis of treated human serum*

Samples of goat anti-human  $\alpha_2\text{M}$  and *S. aureus* coated with anti-mouse immunoglobulin and monoclonal antibodies were incubated with human serum plus inhibitors for 18 h at 4°C. The solutions were spun down and applied to Whatman No. 3 filter paper strips. Horizontal starch gel electrophoresis (Poulik system) was performed as described by Harris and Hopkinson (1976). The gel containing 13% starch (Electrostarch, lot 307) was prepared in gel buffer (76 mM Tris/citrate, pH 8.6) and the bridge buffer, 0.3 M boric acid, brought to pH 8.0 with NaOH. Sample-saturated filter paper strips were inserted into the gel. Electrophoresis was carried out for 6 h at 7 V/cm at room temperature. The gels were stained for protein with 0.02% nigrosin (Sigma).

## Results

Hybridomas were produced in four fusions (Table I) using animals that were immunized with a goat immunoglobulin-human  $\alpha_2\text{M}$  complex. Animals were immunized by an adaptation of the procedure reported by Stahli et al. (1980). Supernatants from these hybridomas were assayed quantitatively by the ELISA method for antibody binding to protein attached to polystyrene plates. Monoclonal antibody activity was detected by peroxidase-conjugated goat anti-mouse immunoglobulin and the appropriate substrate. As shown in Table I, all fusions produced antibodies that reacted with goat immunoglobulin. However, only fusions 1, 3 and 4 produced antibodies with a presumed specificity for human  $\alpha_2\text{M}$ . Fig. 1 illustrates the degree of binding of individual antibodies to equal amounts of both the  $\alpha_2\text{M}$  immunoprecipitate and the antisera alone. These antibodies demonstrate a significantly greater degree of binding to the  $\alpha_2\text{M}$  precipitate than the precipitating

TABLE I

PRODUCTION OF ANTIBODIES AGAINST HUMAN  $\alpha_2$ M-GOAT IMMUNOGLOBULIN COMPLEX

Fusion	Origin of spleen cells	Immunization protocol	Total	Number of hybrids		Hybrids with activity to $\alpha_2$ M after 30 days in culture (%)
				Anti-goat Ig (%)	Anti- $\alpha_2$ M (%)	
1	BALB/c	3 s.c./i.p. 1 i.v.	34	4 (12%)	6 (18%)	0
2	BALB/c	8 s.c./i.p. 3 i.v.	44	2 (4.5%)	0	-
3	BALB/c	8 s.c./i.p. 3 i.v.	313	16 (5%)	39 (12.5%)	18 (46%)
4	Brown Norway	6 s.c./i.p. 3 i.v.	316	41 (13%)	30 (9.5%)	8 (27%)

Abbreviations: i.p. = intraperitoneal; i.v. = intravenous; s.c. = subcutaneous.

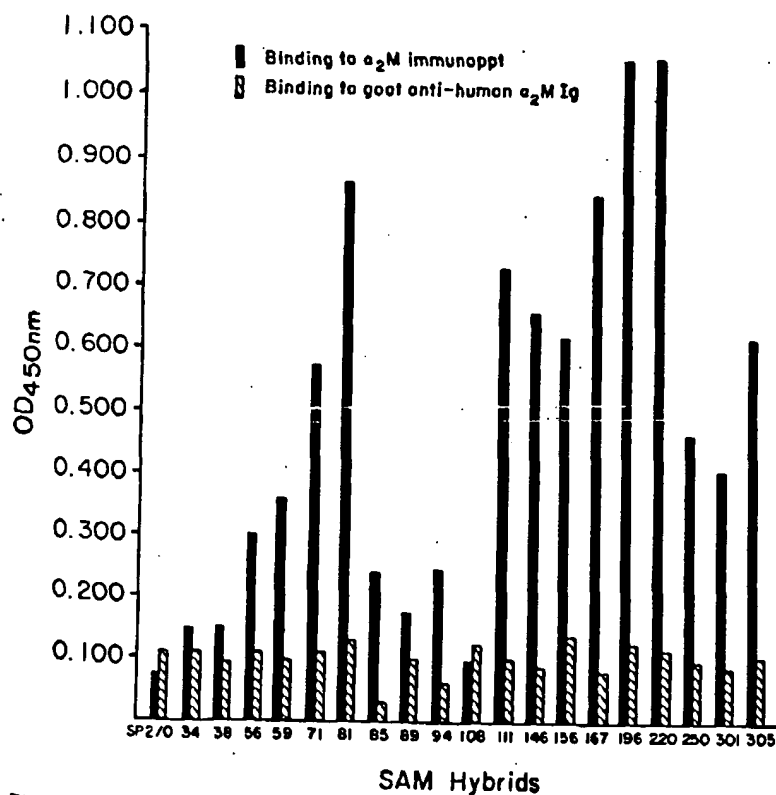


Fig. 1. Binding of mouse anti- $\alpha_2$ -macroglobulin (SAM hybrids) supernatants to immunizing proteins. Bar graphs are representative of duplicate samples from a single assay. Immunoprecipitate and immunoglobulin were bound to wells of a polystyrene plate and tested for the binding of supernatants to the 2 proteins by the ELISA method. Shown here are those monoclonal antibodies that react with the immunoprecipitate but do not bind significantly to goat immunoglobulin.

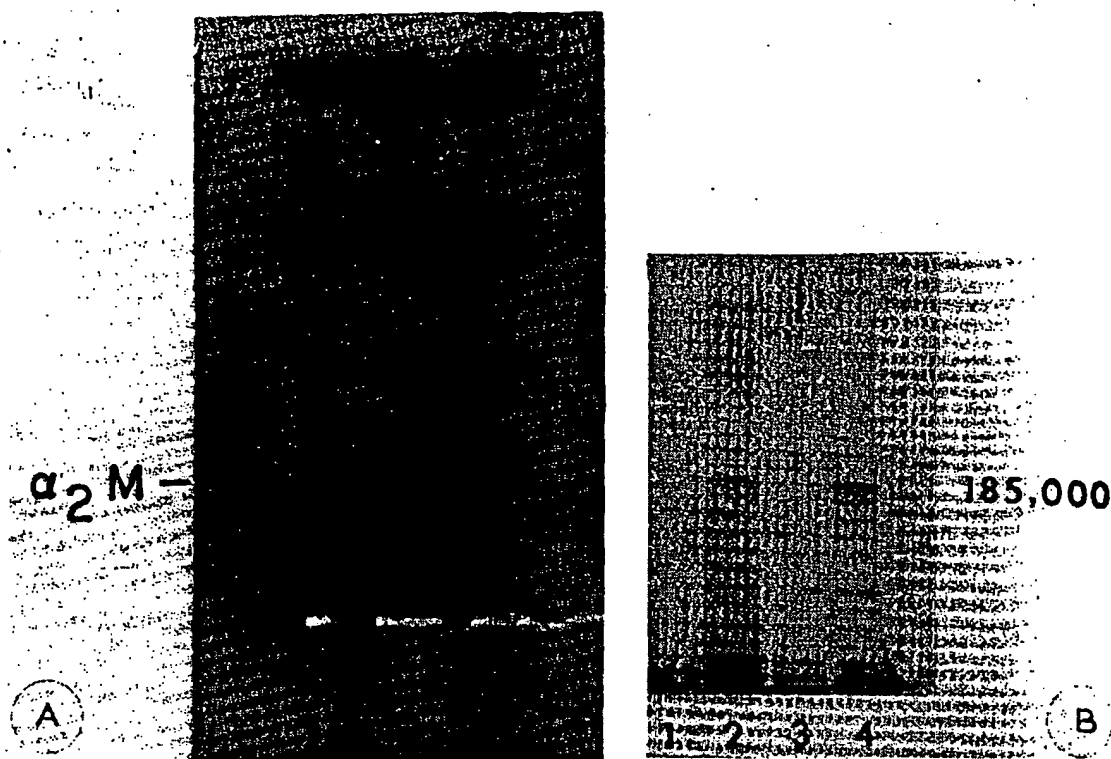


Fig. 2. Confirmation of specificity of anti- $\alpha_2$ -macroglobulin monoclonal antibodies. A: Removal of  $\alpha_2M$  from human serum separated by starch gel electrophoresis. Formalin-fixed *S. aureus* was coated with anti-mouse immunoglobulin, pre-incubated with Sp2/0-Ag14 cell culture supernatant or a pooled sample of 16 anti- $\alpha_2M$  monoclonal antibodies concentrated by ammonium sulfate precipitation and incubated with diluted human serum. Samples were centrifuged and the supernatant applied to a 13% starch gel (Poulik system) using filter paper wicks, electrophoresed for 6 h and stained with nigrosin. Lane 1 is diluted human serum. Lanes 2 and 3 show the proteins remaining in human serum after treatment with pre-coated *S. aureus* that had been incubated with immunoglobulin. Lane 2 is human serum treated with Sp2/0-Ag14 supernatant. Lane 3 is human serum treated with pooled monoclonal antibodies. Note the removal of  $\alpha_2M$  from serum that has been treated with the anti- $\alpha_2M$  monoclonal antibodies. B: Immunoprecipitation of  $\alpha_2$ -macroglobulin from human serum. Formalin-fixed *S. aureus* was coated with goat anti-mouse immunoglobulin and incubated with parental plasmacytoma supernatant or monoclonal antibodies as described above. The antibody-antigen complex was removed from the *S. aureus* by boiling for 2 min with a reducing agent added. *S. aureus* was spun and the supernatant applied to a 5% bis-acrylamide SDS gel. Lane 1 contains the molecular weight standard. The remaining lanes contain the immunoprecipitates found using: pooled supernatants from 16 hybridomas making monoclonal anti- $\alpha_2$  macroglobulin (lanes 2 and 4) and Sp2/0-Ag14 cell culture supernatant (parental plasmacytoma) (lane 3).

antisera. More than 50% of the hybridomas with a detectable reactivity to human  $\alpha_2M$  lost this activity as the cultures were expanded during the 4 weeks following the initial assay (Table I). Hybridomas which did not lose antibody activity during that time were cloned in semi-solid agarose.

The reaction of 16 mouse monoclonal antibodies with  $\alpha_2M$  was confirmed by the immunoprecipitation of the protein. Antibody was pooled from 16 mouse hybridomas and concentrated by ammonium sulfate precipitation before reaction with anti-mouse immunoglobulin-coated *S. aureus*. This coated *S. aureus* was then incubated with human serum to remove the corresponding antigen. After centrifuga-



tion of the *S. aureus*-antibody complex, the supernatant was applied to a 13% starch gel and electrophoresed. Comparison of human serum before and after treatment with the pooled antibodies shows the removal of the protein band corresponding to the migration pattern of  $\alpha_2$ M in starch gel electrophoresis of human serum treated with the monoclonal antibody-*S. aureus* complex (lane 3, Fig. 2A). Alternatively, confirmation of this specificity by SDS-polyacrylamide gel electrophoresis of substances precipitated from human serum treated with monoclonal antibody-*S. aureus* complex demonstrate the precipitation of a single protein with a molecular weight of approximately 185,000 (lanes 2, 4, Fig. 2B). This is the molecular weight of the reduced subunit chain of  $\alpha_2$ M (Harpel, 1973). Further confirmation of the specificity of the monoclonal antibodies was obtained by reaction of the individual monoclonal antibodies which comprised the pooled antibody mixture with purified  $\alpha_2$ M.  $\alpha_2$ M purified from human plasma according to the procedure of Kaplan et al. (1981) was used as the antigen in the ELISA procedure. All antibodies demonstrated significant binding to the purified protein (data not shown).

The antibody class of 18 independent mouse antibodies was determined by immunoprecipitation with class specific antisera. Three hybridomas produce IgM antibodies, 13 produce IgG<sub>1</sub> antibodies, one produces IgM and IgG<sub>1</sub>, and one produces IgG<sub>1</sub> and IgG<sub>2a</sub>. The two hybridomas which produce two classes of antibody were uncloned hybrids. An estimate of the antigenic determinants identified by these antibodies was determined by competitive binding inhibition assays as described in Materials and Methods. These assays indicate that a minimum of 7 sites are identified by 13 monoclonal antibodies (data not shown).

## Discussion

A major advantage in using monoclonal antibodies is that one can isolate a single antigen from a complex mixture. A simple purification of the antigen often simplifies the monoclonal antibody screening procedure. We have demonstrated that the use of conventionally produced antisera is advantageous in isolating antigens that will ultimately be used for the immunization of animals for hybridoma production. The goat immunoglobulin-human  $\alpha_2$ -macroglobulin immune complex acted as an effective immunogen in both rats and mice. Consequently, we were able to demonstrate the successful production of stable monoclonal antibody producing lines with a specificity to human  $\alpha_2$ M.

This method has also been used to produce monoclonal antibodies to human complement components (Jonak, personal communication). Several advantages are apparent in using this technique. First, if a conventional antiserum is already available, tedious purification of the antigen for immunization is eliminated. Second, purification of the antigen for the screening procedure is not necessary since antibodies can distinguish between the immunoprecipitate and the antisera alone. Third, since conventional antisera have been produced against a wide variety of proteins and other biological substances, this technique provides an effective method for the study of these same molecules by monoclonal antibodies.

### Acknowledgements

The authors would like to thank Dr. Harry Harris for his suggestions concerning the starch gel electrophoresis and Dr. Douglas Holsclaw of Hahnemann Hospital for kindly providing human plasma samples.

This work was supported by NSF Grant 26757.

### References

- Engvall, E. and P. Perlmann, 1971, *Immunochemistry* 8, 871.
- Harpel, P.C., 1973, *J. Exp. Med.* 138, 508.
- Harris, H. and D.A. Hopkinson, 1976, *Handbook of Enzyme Electrophoresis in Human Genetics* (North-Holland, Amsterdam).
- Jonak, Z.L., 1980, in: *Monoclonal Antibodies. Hybridomas: A New Dimension in Biological Analyses*, eds. R.H. Kennett, T.J. McKearn and K.B. Bechtol (Plenum Press, New York) p. 405.
- Kaplan, J., F.A. Ray and E.A. Keogh, 1981, *J. Biol. Chem.* 256, 7705.
- Kennett, R.H., K.A. Denis, A.S. Tung and N. Klinman, 1978, in: *Current Topics in Microbiology and Immunology*, Vol. 81, eds. F. Melchers, M. Potter and N.L. Warner (Springer-Verlag, Berlin) p. 77.
- Köhler, G. and C. Milstein, 1975, *Nature (London)* 256, 495.
- Lampson, L.A., 1980, in: *Monoclonal Antibodies. Hybridomas: A New Dimension in Biological Analyses*, eds. R.H. Kennett, T.J. McKearn and K.B. Bechtol (Plenum Press, New York) p. 395.
- Laurell, C.-B. and J.-O. Jeppsson, 1975, in: *The Plasma Proteins-Structure, Function and Genetic Control*, Vol. 1, ed. F.W. Putman (Academic Press, New York) p. 229.
- Shulman, M., C.D. Wilde and C. Köhler, 1978, *Nature (London)* 276, 269.
- Stahli, C., T. Staehelin, V. Miggiano, J. Schmidt and P. Haring, 1980, *J. Immunol. Methods* 32, 297.